DNA barcoding of Indian Sarus Crane (*Grus antigone antigone)* and its conservation prospects

SYNOPSIS

For the award of the degree of Doctor of Philosophy in Zoology

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PhD Research Proposal

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Introduction

The Indian Sarus Crane (ISC) is the only resident breeding crane in India and South East Asia, and is the world's tallest flying bird. It is the State bird of Uttar Pradesh, but has been extirpated from large portions of its historic ranges and continues to decline in areas where it still exists due to huge deterioration in the habitat and quality of life and subsequently the numbers of the avian fauna of the state

The current range of the Indian Sarus Crane includes the plain of Northern, north western and western India and the western half of Nepal's Tarai lowlands. In these areas the major threats include loss and degradation of wetlands – due to agricultural expansion, industrial development, river basin development, pollution, warfare and heavy use of pesticides. Sarus Crane has been hunted in the portions of their historic and present range. Trade may also be a threat in some areas, eggs and chicks are stolen for food and pet trade. This has resulted into the entry of Indian Sarus Crane into the endangered category of IUCN red list under criteria A1b, c, d, e.

An array of research has been conducted on the conservation of Sarus including its taxonomy, historical range, demography, habitats and threats as well but quite a few studies have come up concerning its genetic status which creates a gap between the steps needed and the actual efforts being made. Thus, the underlying objective of this study is to bridge this gap by the means of a DNA Barcode as it is a relatively new and simple approach to taxon recognition. It has become a universally recoverable DNA segment that can be applied as an identification marker across all eukaryotic species. It employs the diversity among the 648 base pair long 5' region of the first sub unit of the Cytochrome Oxidase (COI) gene from the mitochondrial DNA. This gene is chosen for

making the barcode because it is rich in highly conserved primers and the evolution rate of this portion of the gene is rapid enough to differentiate between phylogeographic groups.

DNA barcodes primarily serve the purpose of species identification. Thus, these molecular identifiers are useful for three main purposes in conservation biology : (a) for the identification of illegally used animal or plant products (wildlife forensics), (b) to accelerate the pace of assessment of biodiversity studies which is currently time taking and cumbersome and (c) to help in any ex situ breeding programme of this endangered species.

Therefore, this study intends to focus on building a molecular database of the Indian Sarus Crane using mitochondrial DNA markers for applications in conservation genetics and avian forensics by employing non invasive genetic sampling to develop simple and effective method of genetic analysis.

Review of Literature

This research proposal is an attempt to amalgamate various arenas of biological sciences crucial for future scientific research which include non invasive genetic research, conservation genetics and wildlife forensics. Hence, the literature is categorised accordingly and studied as follows:

Morin *et.al*. in **1994** pioneered non-invasive genetic sampling by employing chelex method for genotyping of birds from feather samples, stating bleeding and biopsy are no longer necessary for genetic sampling. During the same time studies were carried out in **1994** and **1996** to quantify the rates and patterns of cytochrome B evolution in Cranes by **Krajewski *et.al*** who further went on to discover mitochondrial sequence variation in the subspecies of the Sarus Crane differentiating them from the Brolga and White naped crane outgroups. In **1999** non-invasive genetic sampling attracted scientists when **Taberlet *et.al*** carried out genotyping from non invasive samples including bear faeces and in another study explained the two view points about the approach, its shortcomings and ways to overcome it. In **1999 Hasegawa *et.al.*** studied the variation of the mitochondrial control region sequence in the three subspecies of Sarus Crane . Now, newer sources of Non invasive genetic sampling were explored, for example, **Bello, Francino** and **Sanchez** in **2001** devised a DNA extraction method from feathers with RNAse digestion for big feathers. Also**, Morin *et.al*** in **2001**

carried out quantitative PCR analysis of DNA from non invasive samples for accurate microsatellite genotyping of wild chimpanzees. Scientists including **Krajewski *et.al*** sped up research work on Cranes in **2001**, when they studied the mitochondrial phylogeography, subspecific taxonomy and conservation genetics of the Sandhill Crane. **Segelbacher** in **2002** tested the reliability of feather samples for non invasive genetic analysis. He showed that PCR success was higher from extracts of plucked feathers than moulted feathers. The Sarus research pioneers **Sundar** and **Choudhury** in **2003** gave a full fledged report on the history and present status of the Sarus Crane in India which included its number, the biology, the social structure and the threats to its existence. Also, **Archibald**, **Sundar** and **Barzen** in **2003** reviewed the three subspecies of Sarus Crane for their morphology, distribution, habits and habitat, breeding biology and present status. **Desalle** and **George Amato** in **2004** explained the expansion of conservation genetics, describing it as a crisis discipline and genetic approaches to characterize endangered species. Genetic sampling from feathers was further established when **Cruz *et.al*.** in **2005** looked closely at feathers as the non invasive source for genetic analysis and stated in their study that the blood clot in the superior umbilicus of the feather shaft is a better source of DNA than the previously used tip samples from moulted feathers. **Daniel Rubinoff** in **2005** explained the utility of mitochondrial DNA barcodes in species conservation. **DeVolo *et.al*.** in **2008** created an improved extraction method to increase DNA yield from moulted feathers. In **2010 Krajewski *et.al.*** genotyped the complete mitochondrial genome sequence and gave the phylogeny of Cranes. They also gave the list of standard primers for all the genes of the mitochondrial DNA. **Linacre and Tobe** in **2011** gave an overview to the investigative approach to species testing in wildlife forensic science by the aid of cytochrome B and COI gene. Feather genetic research accelerated when **Speller**, **Nicholas** and **Young** in **2011** successfully extracted mitochondrial DNA from as low as two feather barbs for bird species identification in forensic wildlife investigation. **Chen *et.al*.** in **2011** tested the utility of mitochondrial COI for estimates of relationships between crane species. In **2012 Ghorpade *et.al*.** discovered molecular sexing as an important strategy for conservation breeding. **Vergara *et.al*.** in **2014** carried out individual identification and distribution assessment of otters through non-invasive genetic sampling. In **2014 Jha** and **Mckinley** studied the demography and ecology of Indian Sarus Crane in Uttar Pradesh, by diving it into various agro climatic zones. **Goncalves *et.al*.** in

2015 used COI barcodes and 16S DNA to identify illegal parrot trade. **Nasim Ahmad Ansari** studied the population composition and distribution of the Indian Sarus Crane in the Surajpur Wetland of the NCR Region in **2015** The multipurpose nature of DNA barcoding was seen by **Barriera *et.al.*** in **2016** who proved the utility of DNA barcodes in quality assessment beyond the main purpose of species identification. **Jan** and **Fumagalli** in **2016** studied polymorphic DNA markers for forensic identification and parentage analysis of seven threatened species. **Urantowka *et.al.*** in **2017** explained the influence of molecular markers on inferring the phylogenetic relationships between the representatives of the same taxa.

Objectives of the study:

- DNA Barcoding

1. To create DNA standard profile of Sarus Crane for building the genetic database.
2. Identification of the Cytochrome Oxidase sub unit I in the mitochondrial genome.
3. To identify other mitochondrial markers which can also be suitable for studies.

- Conservation Prospects

4. To estimate the genetic diversity, richness and abundance of the Indian Sarus Crane by using various bioinformatic and GPS tools.
5. To assess the nature and magnitude of threat to the Indian Sarus Crane.
6. To develop conservation strategies for Sarus Crane by the aid of conservation genetics in Uttar Pradesh.
7. To recommend building a breeding programme for Sarus Crane.

Protocols

Classification

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Gruiformes

Family: Gruidae

Genus: *Grus*

Species: *antigone*

Sub species: *antigone antigone*

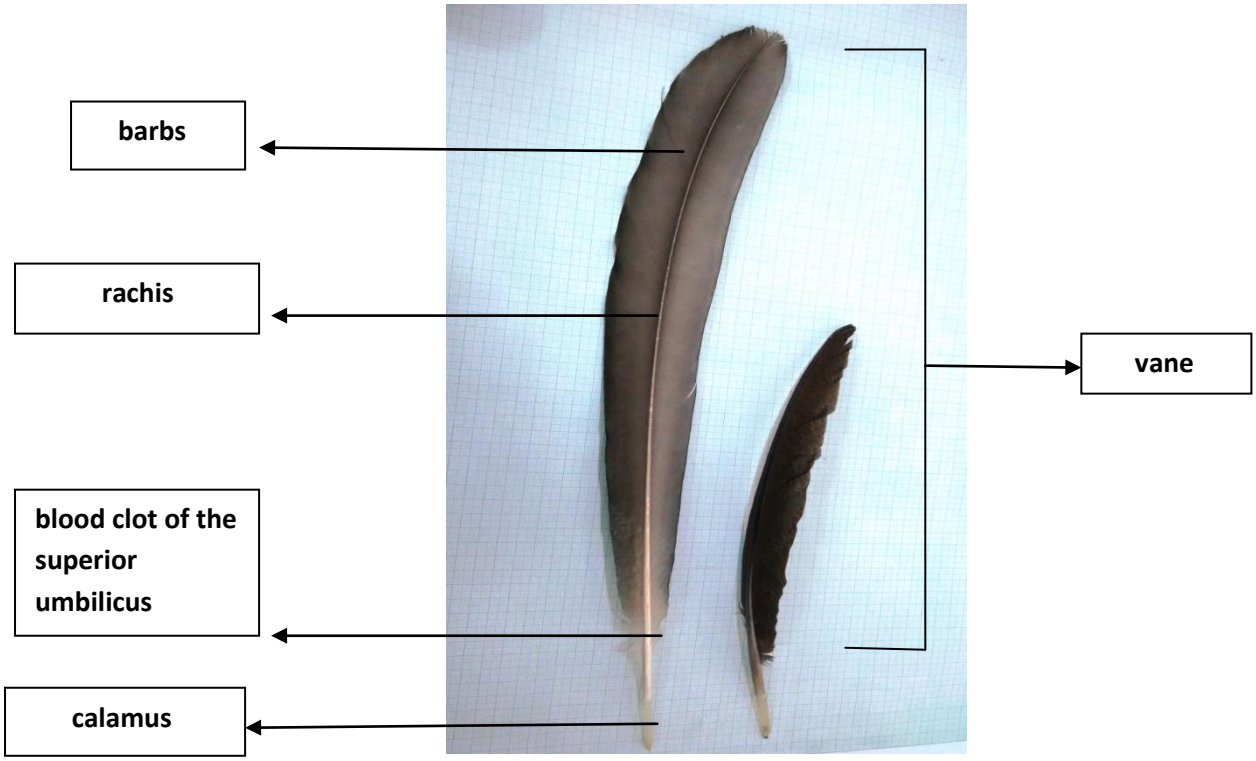
Sample Collection

- The sampling will be done in the wetlands of the South-Western semi arid plains of Uttar Pradesh comprising mainly of the districts of Etawah and Mainpuri.

The Sampling Site

- The sampling site will be divided into various zones and a census along with feather sample collection will be done during the morning and evening hours on the same day for each zone to avoid double counting.
- The counting will be done preferably during the summer season as during this time the Cranes are confined to the wetlands due to immense need of water.
- All feather samples will be collected non invasively which includes only shed feathers and feathers from a dead bird.

DNA Extraction



Parts of a bird feather

- The calamus and the blood clot of the superior umbilicus available in freshly shed feathers will be used for DNA extraction.

Purity Assessment of the Extract

- Spectrophotometric analysis will be done at an O.D. of 260 nm and 280 nm. A ratio of 1.2 to 1.8 will indicate purity of sample.
- Visualisation of genomic DNA bands by agarose gel electrophoresis will be done.

PCR Amplification

- PCR amplification will be done using standard primers given by Krajewski *et.al.* (2010).

Automated Sequencing

- Automated sequencing will be done by ABI PRISM BigDye Terminator Cycler Sequence Ready Reaction Protocol commercially.
- The sequence will be edited using various bioinformatic tools such as Sequence Manipulation suite and ORF Finder and formatted using FASTA and PHYLIP.

Sequence Submission

- The validated sequences will be submitted to GenBank and BOLD using softwares such as Sequin or Barcode Submission Tool.

Population Genetic Studies

- The obtained data sets from the census and the mitochondrial DNA sequences will be subjected to tools such as AMOVA, Arlequin and DNAsp to calculate the richness and diversity of the species and accordingly evolving conservation strategies for the Indian Sarus Crane.

Expected outcomes of the study

After the study is completed we shall be successful in obtaining the following:

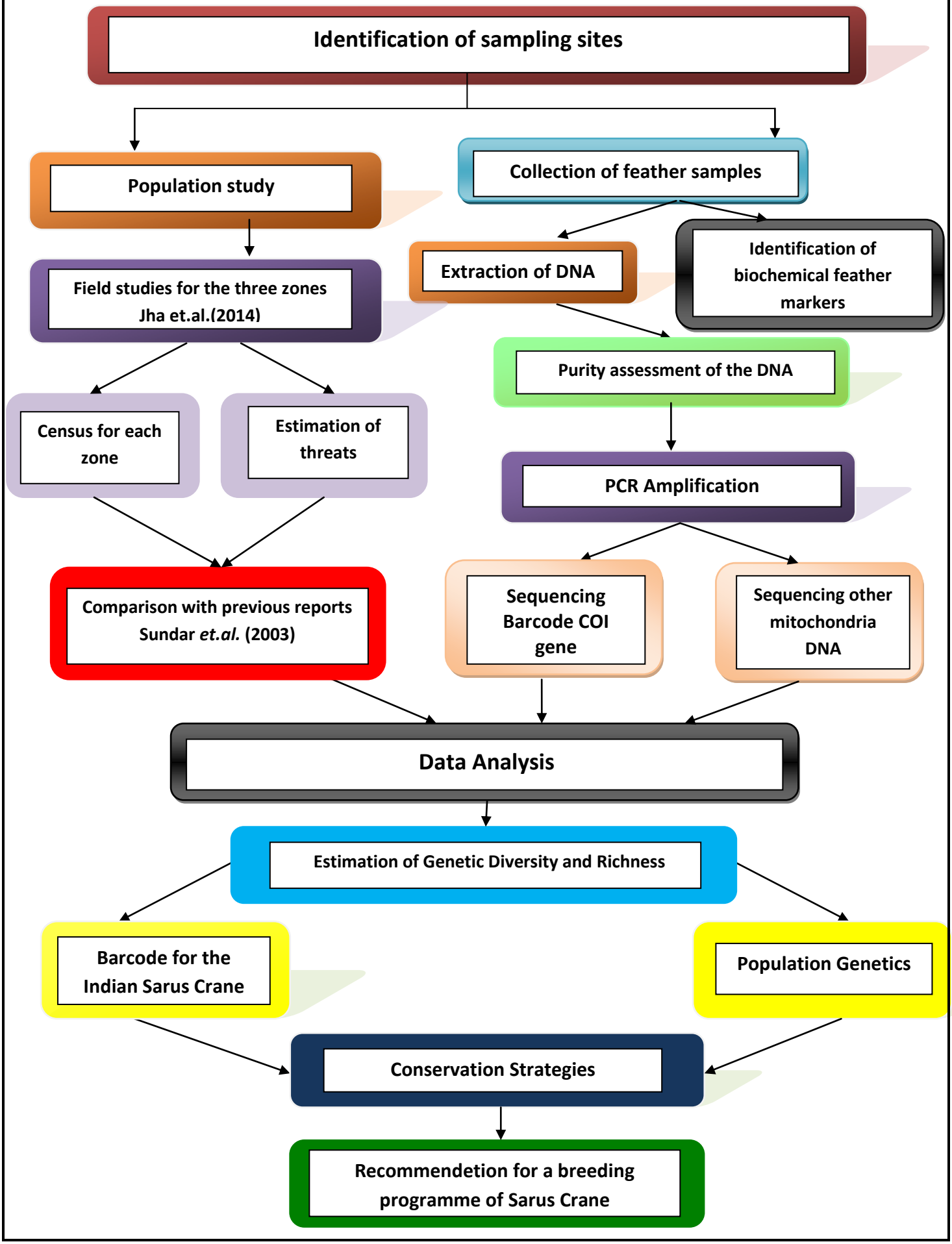
- Newer techniques for deriving DNA samples through non invasive process.
- DNA standard profile of cranes for building the genetic database.

- An account of genetic diversity of the Indian Sarus Crane in Uttar Pradesh
- Current status of Indian Sarus Crane in Uttar Pradesh and conservation strategies for the improvement of the same.

Progress already made

- Sample collection started
- Pilot studies initiated

Plan of Work


Identification of sampling sites
Population study
Collection of feather samples
Field studies for the three zones Jha et.al.(2014)
Extraction of DNA
Identification of biochemical feather markers
Purity assessment of the DNA
Census for each zone
Estimation of threats
PCR Amplification
Comparison with previous reports Sundar *et.al.* (2003)
Sequencing Barcode COI gene
Sequencing other mitochondria DNA
Data Analysis
Estimation of Genetic Diversity and Richness
Barcode for the Indian Sarus Crane
Population Genetics
Conservation Strategies
Recommendetion for a breeding programme of Sarus Crane

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